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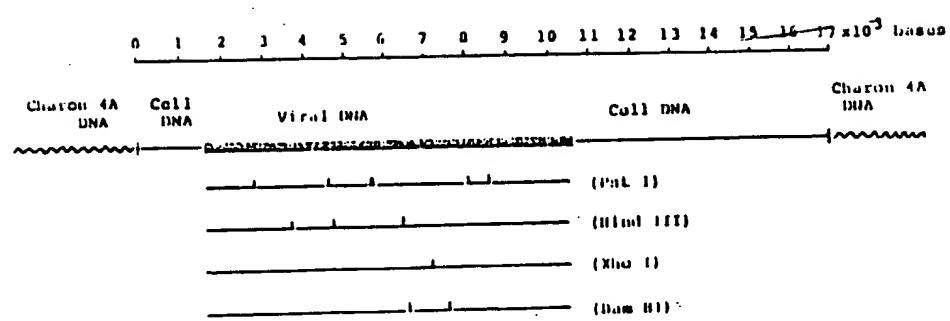
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⑯ DNA complementary to RNA of human leukemia virus.

⑯ Described is a viral genomic DNA and fragments thereof
complementary to genomic RNA of human leukemia virus
and a recombinant DNA molecules containing the genomic
DNA or fragments thereof. The genomic DNA and fragments
thereof and the recombinant DNA molecules are useful for
the diagnosis, prevention and therapy of human leukemia.

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Fig. 1



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DNA COMPLEMENTARY TO RNA
OF HUMAN LEUKEMIA VIRUS

6. Dec. 1983

10 Background of the Invention

It has been reported that human leukemia virus, specifically, adult T-cell leukemia virus (referred to as ATL virus hereinafter) is a retrovirus which is a cause of human adult T-cell leukemia and that the provirus is integrated 15 in the chromosome of tumor cells and cell lines established from leukemia patients (M. Yoshida, et al., Proc. Natl. Acad. Sci., USA, 79, 2031, 1982). Therefore, clarification of the structure of human leukemia virus has been considered to greatly contribute to diagnosis, therapy and prevention 20 of human leukemia and/or lymphoma. As the report so far published on the structure of human leukemia virus, there has been the report by Oroszlan, et al. (Proc. Natl. Acad. Sci., USA, 79, 1291 - 1294, 1982) which discloses the sequence of only 25 amino acids of viral protein p24 of a 25 human leukemia virus called HTLV.

Summary of the Invention

The present inventors have made studies of human 30 leukemia virus gene and succeeded in the cloning of the provirus DNA integrated in leukemic cell DNA, which is complementary to the genomic RNA of ATL virus, and the determination of the sequence of all the bases of the DNA.

The present invention relates to a DNA complementary to RNA of human leukemia virus and a recombinant DNA 35 containing the DNA. The present invention particularly relates to a viral genomic DNA and DNA fragments thereof complementary to genomic RNA of human leukemia virus and

recombinant DNA molecules containing said genomic DNA and DNA fragments respectively. The present invention furthermore relates to the use of said DNA and DNA fragments thereof as diagnostic compositions in the detection of 5 human leukemia and/or lymphoma.

Brief Description of the Drawings

10 Fig. 1 shows the restriction map of λ ATK-1 obtained by the present invention.

Detailed Description of the Invention

15 As the source of DNA complementary to the genome of human leukemia virus, the DNA extracted from leukemia cells of adult T-cell leukemia patients, cell lines established from these leukemia cells, cells infected with human leukemia virus or cultured cells thereof are used. In other cases, the double stranded DNAs are synthesized 20 from the genomic RNA of human leukemia virus using a reverse transcriptase. A preferred example is the DNA which is named ATK-1, consisting of 9,032 base pairs and obtained by extraction from adult T-cell leukemia cells isolated from peripheral blood of adult T-cell leukemia patients.

25 The restriction enzyme map and the entire base sequence of ATK-1 are illustrated in Fig. 1 and Table 1. ATK-1 consists of the following five important parts:

30 (1) LTR : The gene at both ends of the virus gene which is essential for the regulation of virus propagation and plays an important role in insertion of the provirus into the chromosomal DNA of cells. The gene consists of 754 base pairs.

35 (2) cag protein gene : The gene coding for the polypeptides which constitute the inner structure of the virus particle. The gene consists of 1,290 base pairs.

(3) pol gene : The gene coding for a reverse transcriptase enzyme (RNA dependent DNA polymerase) and consisting of 2,688 base pairs. Q113078

5 (4) env gene : The gene coding for the glycoprotein on the surface of the virus particle which is responsible for the infectivity of the virus. The gene consists of 1,464 base pairs.

10 (5) pX-I, pX-II, pX-III and pX-IV : These possible genes have not been clarified in respect of role and consist of 297, 261, 333 and 735 base pairs, respectively.

The above five sequence units of fragments respectively can be used as follows:

15 (1) LTR : Since the sequence unit is essential for the propagation of the virus and responsible for the insertion of the provirus into the cell DNA, the DNA of LTR can be used as a probe for the diagnosis of viral infection with adult T-cell leukemia virus.

20 (2) gag protein gene : Since the proteins encoded by the genes are produced in the largest amount, and the antibodies to these proteins and the antigens thereof are easily detectable, the gene can be used for the diagnosis of adult T-cell leukemia or viral infection.

25 (3) pol gene : Since the gene codes for a reverse transcriptase, a specific inhibitor to the gene or gene product can be used for the prevention of infection and dispersion of the virus.

30 (4) env gene : Since the gene codes for a glycoprotein responsible for the infectivity of the virus, the protein or the glycoprotein can be administered in the form of a vaccine for active immunization. Therefore such gene products are most useful for the prevention of virus infection, specific destruction of leukemia cells and diagnosis of virus infection.

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As illustrated in Fig. 1, ATK-1 has 5, 3, 1 and 2 cleavage sites for the restriction enzymes PstI, HindIII, XhoI and BamHI, respectively.

5 The DNA complementary to the genomic RNA of human leukemia virus can be recovered as a recombinant with a vector DNA. The present invention provides also the recombinant DNA.

10 The recombinant DNA of the present invention can be obtained by extracting DNA from adult T-cell leukemia cells of adult T-cell leukemia patients, cell lines established from these leukemia cells, cells infected with the virus or cultured cells thereof, cutting out the virus gene with restriction enzymes and inserting the gene into a vector DNA by recombinant DNA technology.

15 Adult T-cell leukemia cells are separated from peripheral blood of adult T-cell leukemia patients by the centrifugation method using Ficoll [A. Boyum: Scand. J. Clin. Lab. Invest. 21, 97 (1968)]. The cell lines having the gene of human leukemia virus are obtained by culturing 20 the leukemia cells of an adult T-cell leukemia patient in the presence of a T-cell growth factor (TCGF) for a long period [B. J. Polesz et al., Proc. Natl. Acad. Sci. USA, 77, 6615 (1980)] or by culturing the leukemia cells mixed with lymphocytes in the blood of fetal umbilical cord 25 [I. Miyoshi et al., Nature 294, 770 (1981)]. Cells infected with the virus are also obtained by culturing the cells producing human leukemia virus mixed with human lymphocytes [I. Miyoshi et al., Gann, 72, 997 (1981), N. Yamamoto et al., Science, 217, 737 (1982)].

30 The cell having integrated form of human leukemia virus genes are disrupted by adding 0.5-1.0% SDS, and high molecular weight DNA and RNA are extracted with phenol, etc. The RNA is decomposed by 10-100 µg/ml RNase treatment at 37°C for 0.5-1 hour and removed, followed by recovery of the DNA 35 by phenol extraction. The DNA is cut by the treatment with restriction enzymes such as EcoRI, and DNA fragments of 10-20 Kb are obtained by the purification by phenol extraction. The DNA is subjected to digestion by an appropriate restriction enzyme in the presence of a buffer solution containing

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Tris-HCl (pH 7.0 - 8.5, 10 - 50 mM), $MgCl_2$ (5 - 10 mM), $NaCl$ (0 - 150 mM), mercaptoethanol (0 - 10 mM), and the like.

Separately, phage DNA such as λ Charon 4A or an Escherichia coli plasmid such as pBR322 used as a vector 5 is cut by the treatment with an appropriate restriction enzyme such as EcoRI. The resulting DNA fragments are isolated and purified by agarose gel electrophoresis.

The cell DNA fragment and the vector DNA fragment both of which are cut with the same restriction enzymes are 10 mixed to combine both DNA fragments. The recombination reaction is carried out with T4 DNA ligase in the presence of a buffer solution containing Tris-HCl (pH 7.0 - 8.5, 10 - 50 mM), $NaCl$ (50 - 150 mM), $MgCl_2$ (5 - 10 mM), ATP (0.05 - 0.2 mM) and the like at a temperature of 8 - 15°C for a 15 period of 10 - 48 hours.

In the case where a phage DNA is used as the vector DNA, the recombinant DNA is recovered in the following manner.

The DNA recombined in the above-mentioned manner 20 is reconstructed with λ phage particles by, for example, the method of Blattner, et al. [Science, 202, 1279 (1978)]. Escherichia coli sensitive to λ phage such as DP 50F [D. Piemeister, et al., Nature, 263, 526 (1976)] is infected with the reconstructed λ phage particle and cultured on an agar 25 medium to form plaques of λ phages. Then, a nitrocellulose membrane is stuck on the agar medium to transfer and fix a part of the phages to the membrane. Separately, a ^{32}P labelled cDNA complementary to viral RNA is synthesized by, for example, the method of Yoshida, et al. [Proc. Natl. 30 Acad. Sci., USA, 79, 2031 - 2035 (1982)] using the disrupted virus particles which were purified from the cells producing human leukemia viruses. The (^{32}P)-cDNA as a probe is hybridized with the phage DNA fixed on the nitrocellulose membrane and λ phage recombinants having a gene complementary 35 to human leukemia virus RNA are detected by autoradiography. For plaque purification the procedure described above is preferably repeated twice. Thereafter, the λ phage

insert is isolated and subcloned into the BamHI site 0113078
plasmid pBR322.

In the case where a plasmid DNA is used as the
vector DNA, the recombinant DNA is recovered in the follow-
5 ing manner.

The DNA recombined in the above-mentioned manner
is used to transform Escherichia coli strains such as
Escherichia coli $\chi 1776$ [ATCC 31244, Molecular Cloning of
10 Recombinant DNA, Scott, W.A. & Werner, R., edited. Academic
Press., p.99 - 114 (1977)] and Escherichia coli C600 [R.K.
Appleyard et al., Genetics 39, 440 (1954)] by the method of
Enea, et al. [J. Mol. Biol., 96, 495 - 509 (1975)]. Since
15 the recombinant plasmid carries β -lactamase gene which is
harbored in the vector DNA such as Escherichia coli plasmid
pBR322, the transformed Escherichia coli strains are resist-
ant to ampicillin. Selection of a transformant containing
a novel recombinant DNA harboring a DNA sequence complemen-
tary to a genomic RNA of human leukemia virus from these
ampicillin-resistant strains is carried out by the same
20 colony hybridization method with (^{32}P)-cDNA using a nitro-
cellulose membrane as in the case of phage.

The DNA complementary to RNA of human leukemia
virus is recovered from the thus obtained recombinant DNA
by the method of Maniatis et al. [Cell, 15, 687 (1978)] and the
25 base sequence thereof is determined by the method of Maxam
and Gilbert [Methods in Enzymol., 65, 499 (1980)].

The DNA and recombinant DNA of the present invention
are expected to be very useful for the diagnosis,
prevention and therapy of human leukemia as described below.

30 (1) Methods of diagnosis of human leukemia and/or
lymphoma and virus infection can be established using a
part or the whole of the recombinant DNA.

35 (2) The amino acid sequence of the antigenic proteins
encoded in the viral genome or fragments thereof can be
determined based on the whole base sequence, and peptides
or proteins containing the whole or a part of the amino acid

sequence can be synthesized and produced in a large amount.

5 (3) Genomic DNA or fragments thereof inserted in the recombinant is reinserted in another vector DNA and propagated and amplified in bacteria or eukaryotic cells, whereby virus antigenic proteins can be produced in large amounts.

10 (4) The peptides or proteins produced in 2) and 3) above themselves and the antibodies against them can be used for the diagnosis, therapy and prevention of human leukemia.

15 Examples of the present invention are described below.

15 Example 1

Cloning of integrated proviral genome of ATLV:

In this example, 0.1 ml of heparin injection as an anti-coagulant was added to 5 ml of peripheral blood taken from an adult T-cell leukemia patient for examination. The mixture was gently layered on 3 ml of Ficoll-Conray solution layer [product of Daiichi Kagaku Co.] and subjected to centrifugation at 1500 rpm (1,200 x g) for 30 minutes to recover leukemia cells (about 10^8) separated from erythrocytes. The leukemia cells are lysed in SDS (sodium dodecyl sulfate) with 1% final concentration. 200 μ g/ml proteinase K (product of Merck & Co., Ltd.) was added and the mixture was incubated at 45°C for 2 hours. Subsequently, phenol extraction was carried out three times to obtain about 0.5 mg of a high molecular weight DNA.

25 30 35 250 μ g of the thus obtained DNA was dissolved in a buffer solution (pH 7.5) consisting of 10 mM Tris-HCl, 6 mM $MgCl_2$, 50 mM NaCl and 6 mM mercaptoethanol. 100 units of EcoRI (sold by Takara Shuzo Co.) was added and the mixture was allowed to react at 37°C for 16 hours. The digested DNA is recovered and purified by phenol extraction carried out twice. The DNA was separated in a preparative style by 1% agarose gel electrophoresis and the fractions

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containing DNA fragments corresponding to a molecular size of about 11,000 to 15,000 base pairs were subjected to electrophoretical elution. Thereafter impurities were removed by phenol extraction to obtain a purified DNA.

5 Thus, about 20 μ g of a cellular DNA fragment was obtained.

Separately, 50 μ g of λ phage Charon 4A DNA [F.R. Blattner, Science, 196, 161 (1977)] was dissolved in 100 μ l of the buffer solution mentioned above. 50 units of EcoRI was added and the mixture was allowed to react at 37°C for 10 2 hours, whereby the phage DNA was cut into four DNA fragments. These fragments were separated in a preparative style by 1% agarose gel electrophoresis and two bands of larger sizes (23 Kb and 11 Kb) were cut out, followed by recovery of DNAs by the same method mentioned above. About 15 μ g of phage DNA fragments was obtained from the two bands combined.

About 1 μ g of the cellular DNA fragments and about 2 μ g of the Charon 4A DNA fragments obtained as mentioned above were added to 40 μ l of a buffer solution (pH 7.5) 20 containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.1 M NaCl and 0.1 mM ATP. To the mixture was added 3 units of T4 DNA ligase [product of Bethesda Research Laboratories (referred to as BRL hereinafter)] and the mixture was allowed to react at 12°C for more than 18 hours.

25 The reaction mixture was then subjected to reconstruction with λ phages by the method of Blattner, *et al.* The reconstructed λ phages were added in combination with an indicator bacterium Escherichia coli DP 50F to an agar medium (pH 7.5) containing 10 g/l tryptone, 50 mg/l thymidine, 50 mg/l diaminopimelic acid, 2.5 g/l NaCl and 10 g/l agar, and culturing was carried out. A nitrocellulose membrane (sold by Schleicher & Schüll Co.) was stuck on the agar medium to transfer a part of phage in the plaques to the membrane and the phage DNAs were fixed by heating at 30 35 80°C in vacuo for 120 minutes.

On the other hand, human leukemia virus particles (about 1 mg/ml as protein) purified from about 500 ml of

the culture medium of the MT-2 cells which produce human leukemia cell virus [I. Miyoshi, *Nature*, 294, 770 (1981)] were added to 200 μ l of a reaction solution consisting of 5 50 mM Tris-HCl, 10 mM MgCl₂, 1 mM dithiothreitol, 1 mM dATP, 1 mM dGTP, 1 mM dTTP, 5 μ M ³²P-dCTP, 50 μ g/ml actinomycin D (product of Sigma), 0.02% NP40 detergent (product of Sigma) and 0.5 μ g/ml oligo dT, and reaction was carried out at 37°C for 16 hours, whereby virus ³²P-cDNA complementary 10 to human leukemia virus RNA was synthesized. The ³²P-cDNA (5 x 10⁶ cpm) was obtained after purification by alkaline treatment, phenol extraction, and Sephadex G-100 column chromatography. The ³²P-cDNA was then hybridized with the λ phage DNA fixed on the nitrocellulose membrane and a recombinant phage λ ATK-1 having the genome complementary to 15 RNA of human leukemia virus was detected by radioautography.

Example 2

Preparation of insert DNA (15 Kb) for subcloning, restriction enzyme analysis and sequencing:

20 In this example, Escherichia coli DP 50F was cultured overnight in 10 ml of NZY medium consisting of 10 μ g/l NZ amine, 5 μ g/l yeast extract, 1 g/l magnesium sulfate, 50 μ g/l diaminopimelic acid, 50 mg/l thymidine and 5 μ g/l NaCl. 10⁶ PFU/ml recombinant phage λ ATK-1 obtained 25 in Example 1 was added to the medium and culturing was further carried out overnight. The supernatant fluid of the cultured medium was used as a stock of recombinant phage. 10 ml of the stock was added to 2 l of NZY medium and culturing was carried out at 37°C overnight. Propagated 30 recombinant phages were purified by conventional CsCl density equilibrium centrifugation, followed by phenol extraction to obtain about 400 μ g of recombinant phage DNA. The DNA was completely digested with EcoRI and subjected to 1% preparative agarose gel electrophoresis to obtain 35 about 70 μ g of inserted DNA fragment of about 15,000 base pairs.

The DNA fragment was cut with restriction enzymes such as SalI, HindIII, BamHI, PstI, SmaI, Sau3A, Hinfl and

HpaII and the ends of the cut fragment were labelled with ^{32}P to determine the base sequence of the fragment according to the method of Maxam and Gilbert [Method in Enzymology, 65, 499 (1980)]. The procedures of the determination were repeated to determine the whole base sequence of a human leukemia virus gene. The result is illustrated in Table 1 which shows clearly that the human leukemia virus consists of 9,032 base pairs.

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Table 1. (1)

Table 1 (2)

Table 1 (3)

Table 1. (4)

Table 1 (5)

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About 3 μ g of ATK-1 DNA was completely digested with 10 units of BamHI (product of BRL). This digest generates 3 fragments which were isolated and subcloned into pBR322 separately. A half of the digest (1.5 μ g) was 5 mixed with about 0.5 μ g of pBR322 DNA digested with BamHI as mentioned above and the remaining half was mixed with about 0.5 μ g of pBR322 DNA digested with the enzymes EcoRI and BamHI. The mixtures were subjected to reaction with T4 DNA ligase for 4 days. Escherichia coli C600 was transformed 10 with each reaction solution by the method of Enea et al. [J. Mol. Biol., 96, 495 - 509 (1975)] and plasmids wherein three DNA fragments produced by BamHI treatment are 15 cloned in pBR322 respectively were obtained as illustrated at the bottom of Fig. 1. The plasmid having the 5' terminal DNA fragment was named pATK03, the one having the middle fragment pATK06, and the one having the 3' terminal DNA fragment pATK08. Each plasmid was incorporated in Escherichia coli C600 strain and the strains having the plasmids 20 were deposited on November 23, 1982 with the American Type Culture Collection in U.S.A. as ATCC 39244, 39245 and 39246, respectively.

Example 3

Use of ATK-1 for the diagnosis of human leukemia: 25
In this example, about 10 ml of the peripheral blood was obtained from leukemia patients and 0.5 ml of 10% SDS was added to dissolve the erythrocytes and lymphocytes. 0.15 ml of 100 mM EDTA and 400 μ g of proteinase K were added and the mixture was allowed to react at 45°C for 2 hours. 30 The reaction solution was subjected to phenol extraction three times to extract DNA and the high molecular weight DNA was recovered by winding round a glass rod. Yield was about 50 μ g. 5 μ g of the obtained DNA was digested with 5 units of EcoRI by the method mentioned above. The 35 digest was subjected to 1% agarose gel electrophoresis and DNA fragments were fixed on a nitrocellulose by the method of Southern [J. Mol. Biol., 98, 508 (1975)].

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The novel recombinant phage DNA (λ ATK-1) obtained by the present invention was labelled with a nick translation kit (product of Amersham) in the presence of 32 P-dCTP. The labelled DNA probe was hybridized with the cellular 5 DNA fragments on the nitrocellulose membrane mentioned above and the human leukemia virus genome was detected by autoradiography.

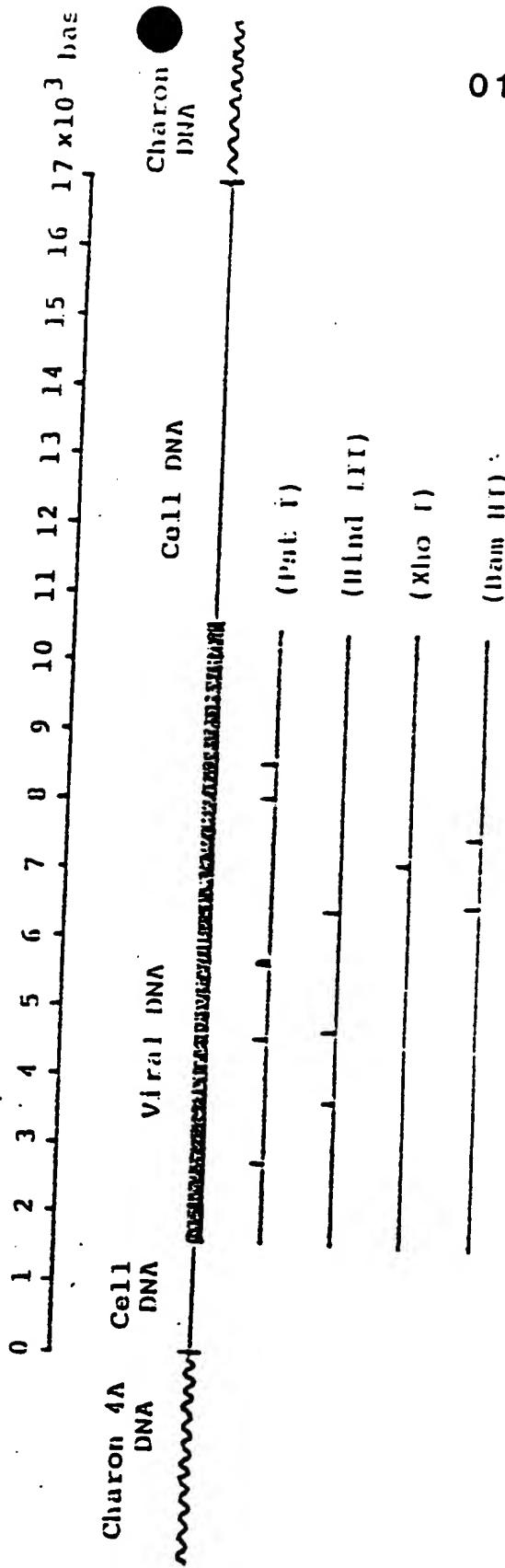
By the method described above, the human leukemia virus genome was detected in all the 18 cases diagnosed 10 pathologically and clinically as adult T-cell leukemia.

WHAT IS CLAIMED IS:

- (1) A viral genomic DNA and fragments thereof complementary to genomic RNA of human leukemia virus.
- (2) The DNA according to claim 1, wherein the human leukemia virus is an adult T-cell leukemia virus (referred to as ATLV hereinafter) or a virus analogous thereto.
- (3) The DNA according to claim 2, wherein the genomic DNA consists of at least 9032 base pairs and has five restriction enzyme sites for PstI, three for HindIII, one for XbaI and two for BamHI.
- (4) The DNA according to claim 3 the entire base sequence of which is illustrated in Table 1.
- (5) A novel recombinant DNA harboring a viral genomic DNA or fragments thereof complementary to RNA of human leukemia virus.
- (6) The recombinant DNA according to claim 5, wherein the human leukemia virus is ATLV or a virus analogous thereto.
- (7) The recombinant DNA according to claim 5, wherein the viral genomic DNA consists of at least 9032 base pairs and has five restriction enzyme sites for PstI, three for HindIII, one for XbaI and two for BamHI.
- (8) The recombinant DNA according to claim 5, wherein the recombinant DNA is constructed of a plasmid DNA derived from Escherichia coli.
- (9) The recombinant DNA according to claim 5, wherein the recombinant DNA is constructed of a phage DNA which propagates in Escherichia coli.
- (10) The recombinant DNA according to claim 5 which has the cleavage sites for restriction enzymes shown in Fig. 1.

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Fig. 1





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**DECLARATION PURSUANT TO RULE 28, PARAGRAPH 4,
OF THE EUROPEAN PATENT CONVENTION**

The applicant has informed the European Patent Office that, until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, the availability of the micro-organism(s) identified below, referred to in paragraph 3 of Rule 28 of the European Patent Convention, shall be effected only by the issue of a sample to an expert.

IDENTIFICATION OF THE MICRO-ORGANISMS

Accession numbers of the deposits: ATCC 39244, 39245 and 39246